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Role of Ca^{++}-Dependent Proteases and Lysosomal Enyzmes in Postmortem Changes in Bovine Skeletal Muscle

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ABSTRACT

Bovine longissimus muscles excised at slaughter were compared to cross-sectional slices of contralateral longissimus muscles obtained 12 hr postmortem for calcium-dependent protease (CDP), cathepsins B, H and L activity, and myofibrillar fragmentation index (MFI). Slices were suspended in either Tris-acetate buffer, buffer + ethylene diaminotetraacetic acid (EDTA), buffer + ethylene glycol-bis (β, aminoethyl ether) N, N, N′, N′-tetraacetic acid (EGTA) or buffer + $CaC1_2$ for either 1, 3, or 7 days while the postmortem associated changes were followed. EDTA, EGTA and Ca^{++} had no effect on cathepsin B, H or L activities. EDTA and EGTA treated slices had 149% whereas Ca^{++} 48% of control CDP activity. Postmortem changes were completed after 24 hr of Ca^{++} treatment but did not occur in EDTA- and EGTA-treated slices. Thus, the changes observed during postmortem storage appeared to be associated with CDP activity rather than catheptic enzymes.

INTRODUCTION

TENDERNESS is the predominant quality determinant and probably the most important sensory characteristic of meat (Weir, 1960; Lawrie, 1966). Currently, postmortem aging appears to be one of the best methods for producing tender beef. Although the increase in meat tenderness is measurable both subjectively and objectively, the mechanism(s) of postmortem tenderization still remains an unresolved issue. If the mechanism of postmortem tenderization is understood, then perhaps it would be possible to advantageously manipulate the process.

Although the exact mechanism of postmortem tenderization still remains a controversial issue, there appears to be a general agreement that proteolysis of myofibrillar proteins is the major contributor to tenderization of beef during postmortem storage (Goll et al., 1983a; Tarrant, 1987). Of the proteases indigenous to skeletal muscle, calcium-dependent proteases (CDPs) and lysosomal enzymes appear to be the best candidates for bringing about the tenderness changes during postmortem storage (Dutson, 1983; Goll et al., 1983a; Etherington et al., 1987; Tarrant, 1987).

Work during the past several years (Koohmaraie et al., 1986, 1987, 1988) has led us to believe that of these two classes of proteases, CDPs appear to be responsible for bringing about postmortem changes. The purpose of these experiments was to test the association of CDPs and lysosomal enzymes under various incubation conditions *in vitro* established to enhance or preclude the role of calcium in postmortem tenderization process.

MATERIALS & METHODS

Sampling procedures

Four cows were slaughtered according to normal procedures. Immediately after slaughter, samples from the longissimus muscle (LD) of one side were removed for determination of Ca^{++}-dependent proteases (CDPs) activity, lysosomal enzyme activities and myofibril fragmentation index (MFI).

At 12 hr postmortem, the entire LD was removed from the other side and 3-5 mm thick cross sectional slices were made. Slices were randomly assigned to the following treatments: (1) buffer (100 mM Tris-acetate pH 7.2, containing 60 mM KCl, 1 mM deoxycholate and 1 mM NaN_3; (2) buffer + 10 mM ethylene diaminotetraacetic acid (EDTA); (3) buffer + 10 mM ethylene glycol-bis (β, aminoethyl ether) N,N,N′,N′-tetraacetic acid (EGTA) and (4) buffer + 10 mM $CaCl_2$. Deoxycholate was included to increase the permeability of the sarcolemma and to facilitate transfer of active compounds between the incubation buffer and the myofibrils Sodium azide (NaN_3) was included to prevent bacterial growth during the experiment. The muscle slices were tied securely by means of thread from one end and placed inside a glass jar (1000 mL total volume). The muscle slices were completely immersed by filling glass jars with the appropriate solution. After 24 hrs, the solutions were poured off and replaced with new solutions. There were two replicates/treatment/animal, each replicate contained three slices for days 1, 3 and 7 analysis of MFI, SDS-PAGE of isolated myofibrils and phase microscopy. In addition, other slices were assigned to each of the above treatments for the determination of CDPs and lysosomal enzyme activities at 24 hr post-treatment time. All incubations were carried out at 2–4°C. At 12 hr postmortem (slices preparation time) MFI, CDP, and lysosomal enzyme activities were determined to document these characteristics of the muscle prior to beginning of the treatments.

Lysosomal enzyme activities

A crude muscle homogenate was prepared from 10g muscle according to the procedure described by Moller et al. (1977). The unsedimentable and sedimentable fractions were then assayed for cathepsins B, H and L according to the procedure described by Barrett (1980) as modified by Kirschke et al. (1983).

Ca^{++}-dependent proteases activities (CDPs)

CDPs were extracted from 100g of muscle according to the procedure described by Koohmaraie et al. (1984c). EDTA concentration in extraction buffer was increased to 20 mM, to prevent autolysis of CDPs during extraction (Hathaway et al., 1982; Mellgren et al., 1982; Goll et al., 1983b), for $CaCl_2$ treatment. Activities of CDPs were determined according to Koohmaraie et al. (1984c).

Myofibril fragmentation index (MFI)

MFI was determined according to the procedure described by Culler et al. (1978).

Myofibril isolation

Myofibrils were isolated according to the procedure described by Goll et al. (1974b).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was conducted according to the procedure described by Porzio and Pearson (1977).

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Protein concentration

Protein concentrations were determined by the biuret method (Gornall et al., 1949).

RESULTS

RESULTS for the effect of treatments on myofibril fragmentation are presented in Table 1. These results clearly indicated that the myofibril fragmentation process was Ca^{++}-mediated. This conclusion was based on the observation that MFI did not change when muscle slices were incubated in the buffer containing EDTA (chelator of divalent ions) and EGTA (chelator of Ca^{++} ions) and that myofibril fragmentation was significantly accelerated when slices were incubated in the buffer containing $CaCl_2$ (Table 1). When slices were incubated with buffer alone, the magnitude of increase in MFI was much less than nonincubated control muscle samples. This difference in MFI values between control and slices incubated in buffer solution could be due to the dilution of intracellular Ca^{++} concentration by the large volume of the buffer.

To examine the effect of these treatments on the degree of fragmentation and integrity of Z-lines, myofibrils prepared from the same preparation used for determination of MFI after 24 hr post-treatment were examined by phase microscopy (Fig. 1 and 2). These results clearly substantiated those of MFI values because Z-lines were intact in nonincubated control muscle samples and in the slices incubated with buffer alone or buffer + EGTA (Fig. 1a, b and Fig. 2a; because the same results were obtained for EDTA- and EGTA-treated slices, for phase microscopy examinations and for the SDS-PAGE pattern of myofibrillar proteins, only those for EGTA are shown) whereas in slices incubated with buffer containing $CaCl_2$, myofibrils were not only highly fragmented but also had lost their Z-lines (Fig. 2b).

To examine the effect of these treatments on the SDS-PAGE pattern of myofibrils, myofibrils were purified after 1, 3 and 7 days incubation (Fig. 3). In the case of nonincubated control muscle (Fig. 3, lanes 1, 5 and 9), there was a gradual increase in the intensity of the 30,000 dalton component accompanied by a gradual disappearance of desmin. A similar pattern was observed when slices were incubated with buffer alone (Fig. 3, lanes 2, 6, and 10). When slices were incubated in the presence of EGTA and EDTA, however, neither of these changes took place (Fig. 3, lanes 3, 7 and 11). On the other hand, when slices were incubated in the presence of $CaCl_2$, both of these changes had occurred by 24 hrs of incubation (Fig. 3, lanes 4, 8 and 12).

To examine the effect of these treatments on the activities of calcium-dependent proteases and lysosomal enzymes, the activities of these proteases were determined at the beginning of the incubation period and 24 hrs after incubation. With the exception of cathepsin H (unsedimentable fraction) and cathepsin L (unsedimentable fraction), catheptic enzyme activities were not affected by the treatments (Table 2). As in the case of CDP, once activated, lysosomal enzymes will undergo autolysis. Therefore, if activated, one would expect to have reduced activity for that particular treatment. Although there was a treatment effect on unsedimentable fractions of cathepsins H and L, they could not be responsible for the observed changes; since where the least activity was observed (EDTA- and EGTA-treated slices) no postmortem aging occurred. On the other hand there was a marked effect on the activities of the CDPs (Table 3). CDPs are dependent on Ca^{++} for activation. Upon activation, CDPs will undergo autolysis and, therefore, if CDPs were activated by any of the incubation conditions, one would expect to have reduced activity of CDPs for that particular treatment. In the absence of Ca^{++}, CDPs will not be activated and the activity of CDPs would be preserved due to lack of autolysis. Results presented in Table 3 show that in slices incubated with Ca^{++} chelators (i.e., EDTA and EGTA), the activity of CDPs was preserved. On the other hand, slices incubated with $CaCl_2$ had significantly less CDP activity than any of the other treatments indicating Ca^{++}-dependent activation and autolysis of CDPs.

DISCUSSION

PROTEOLYSIS of myofibrillar protein appears to be a major contributor to the tenderization process during postmortem storage (Dutson, 1983; Goll et al., 1983a; Tarrant, 1987). Of the proteases indigenous to skeletal muscle, calcium-dependent proteases (CDPs) and lysosomal enzymes appeared to be the best candidates for bringing about the increase in tenderness during postmortem storage.

CDPs have been implicated to be important in postmortem tenderization of beef (Goll et al., 1974a). In spite of remarkable resemblance between the effect of CDPs and postmortem storage on myofibrils (Z-disk weakening, disappearance of troponin-T and appearance of a 30,000 dalton component, disappearance of desmin and titan and no effect on actin and myosin), several legitimate questions have been raised suggesting that CDPs may not be responsible for the changes observed during postmortem storage (Goll et al., 1983a). Work during the past several years (Koohmaraie et al., 1986, 1987, 1988) has led us to believe that CDPs appear to be responsible for bringing about postmortem changes. Koohmaraie et al. (1986) have documented that low calcium requiring CDP (CDP-I) retained 24–28% of its activity of pH 5.5–5.8 and 5°C (postmortem conditions) and more importantly this level of activity was sufficient to reproduce most of the known changes associated with the tenderization process during postmortem aging. Thus, it was possible that CDP-I could be responsible for the postmortem tenderization of beef carcasses. To test this hypothesis, the postmortem changes in three different bovine muscles within the same carcass were examined (Koohmaraie et al., 1988). It is logical to assume that the class of proteases responsible for postmortem aging should have higher activity in the muscle with high aging response, i.e., tenderization and vice versa. These results (Koohmaraie et al., 1988) indicated that regardless of the magnitude of the aging response, the activities of lysosomal enzymes were basically similar in all three muscles. However, in the case of CDPs, their activity followed the same pattern as the aging response. Longissimus muscles which had the highest aging response also had the highest CDP-activity. The Psoas major had the least aging response and CDP-I activity, whereas the biceps femoris muscle was intermediate in both. If indeed our hypothesis is correct and CDP-I is responsible for postmortem aging, then its inactivation, or postmortem handling of the carcasses to provide unfavorable conditions for its activation should prevent the

Table 1—Effect of treatments on MFI ($A_{540} \times 200$)[d]

	Control	Buffer	Buffer + EDTA	Buffer + EGTA	Buffer + $CaCl_2$
0 hr	26.9 ± 2.4	–	–	–	–
12 hr	34.2 ± 0.8	–	–	–	–
Day 1	46.2 ± 1.3[b]	42.3 ± 1.7[b]	33.2 ± 1.5[a]	32.6 ± 2.0[a]	70.4 ± 1.3[c]
Day 3	50.4 ± 1.6[b]	47.9 ± 1.7[a]	28.9 ± 1.7[a]	31.9 ± 1.8[a]	73.4 ± 2.9[c]
Day 7	63.5 ± 2.2[c]	51.0 ± 0.8[b]	30.25 ± 1.0[a]	29.5 ± 1.6[a]	75.3 ± 1.9[c]

[a,b,c] Means in a same row with different superscript differ ($P<0.05$)

[d] Mean ± SEM.

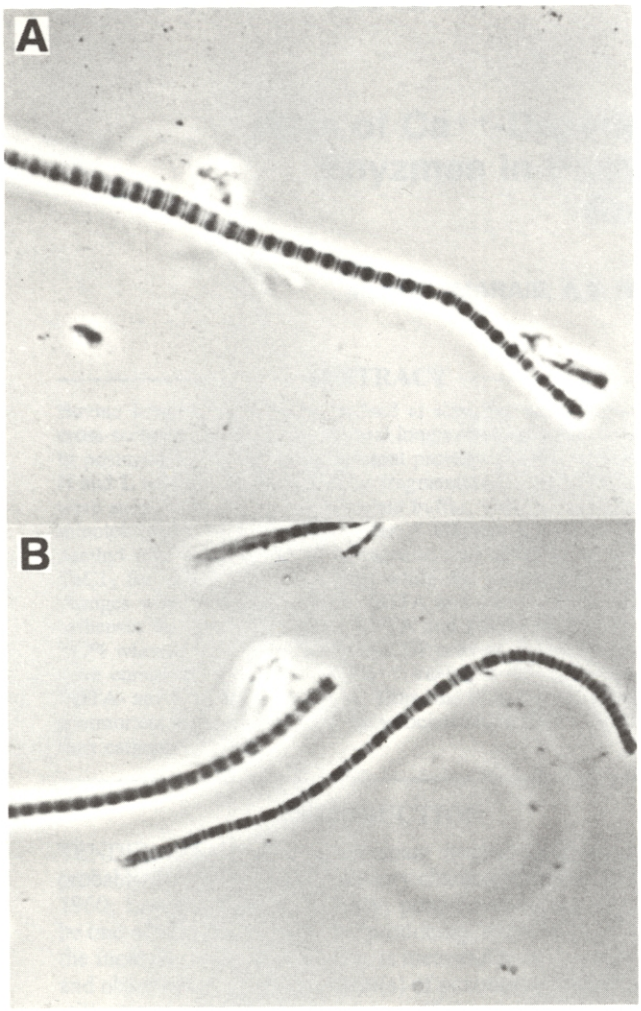

Fig. 1.—Phase micrograph of myofibrils from control at 36 hr postmortem and slices incubated with buffer alone (100 mM tris-acetate pH 7.2, containing 60 mM KCl, 1 mM deoxycholate and 1 mM NaN_3) for 24 hr 2–4°C (X 2000). A = control; B = buffer alone.

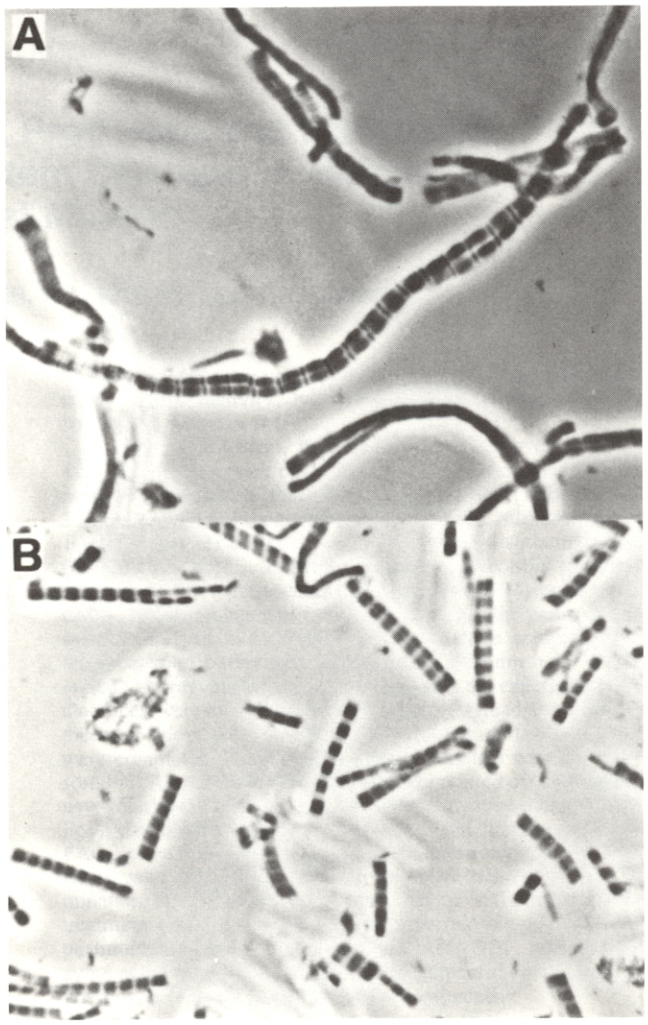

Fig. 2.—Phase micrograph of myofibrils from slices incubated for 24 hr at 2–4°C with buffer (100 mM tris-acetate pH 7.2, containing 60 mM KCl, 1 mM deoxycholate and 1 mM NaN_3) containing 10 mM EGTA and buffer containing 10 mM $CaCl_2$ (X 2000). A = buffer + 10 mM EGTA; B = buffer + 10 mM $CaCl_2$.

postmortem aging. Likewise, generation of favorable conditions for its activation should enhance postmortem changes and the consequential tenderization process.

This study reports experiments that create conditions to prevent activation of CDPs as well as conditions to favor activation of CDPs. The Ca^{++} chelators EGTA and EDTA were used to generate unfavorable conditions for CDP activation because CDPs require Ca^{++} for activation. If CDPs are involved in postmortem tenderization, there should not be any postmortem changes in the presence of Ca^{++} chelators. To create favorable conditions for activation of CDPs, $CaCl_2$ was added as a source of Ca^{++} for activation of CDPs. Because Ca^{++} chelators do not effect lysosomal enzyme activities, postmortem changes in the presence of EGTA and EDTA would indicate that lysosomal enzymes are responsible for postmortem tenderness. If postmortem changes do not occur in the presence of EGTA and EDTA, it can be concluded that CDPs are responsible for postmortem tenderization.

The results presented here clearly indicated that postmortem changes in the myofibril were Ca^{++}-dependent processes. This conclusion is based on the following observations: (1) the MFI changes were accelerated when slices were incubated with a solution containing $CaCl_2$, and these changes did not take place when slices were incubated with solutions containing Ca^{++} chelators (Table 1); (2) the appearance of the 30,000 dalton component which is the most consistent change with postmortem storage (MacBride and Parrish, 1977; Olson et al., 1977; Penny, 1980; Samejima and Wolf, 1976; Yamanoto et al., 1977; Koohmaraie et al., 1984a, b, c; 1986) and disappearance of desmin (Robson and Huiatt, 1983; Young et al., 1981; Koohmaraie et al., 1984a, b, c) occurred within 24 hr of incubation in the presence of $CaCl_2$ and did not take place in the presence of Ca^{++} chelators (Fig. 3). Because incubation of slices in EDTA and EGTA had no effect on the activities of cathepsins B, H, L (Table 2) and postmortem changes did not take place in the presence of EDTA and EGTA (Table 1, Fig. 1, 2, and 3), it is concluded that lysosomal enzymes did not play any role in the changes in myofibril and myofibrillar proteins in this experiment. On the other hand, because incubation of slices in the presence of $CaCl_2$ accelerated postmortem changes (Table 1 and Fig. 1, 2, and 3) and activated Ca^{++}-dependent proteases (Table 3), it is concluded that CDPs have a significant role in the postmortem tenderization of meat.

Etherington et al. (1987) recently reported no relationships between muscle conditioning (aging) rate and the activities of several lysosomal enzymes on Ca^{++}-dependent proteases. Their conclusions regarding lysosomal enzymes supports our conclusion, but contradicts our conclusion regarding the role of CDPs in postmortem tenderization.

The next step in our efforts to understand the mechanism of

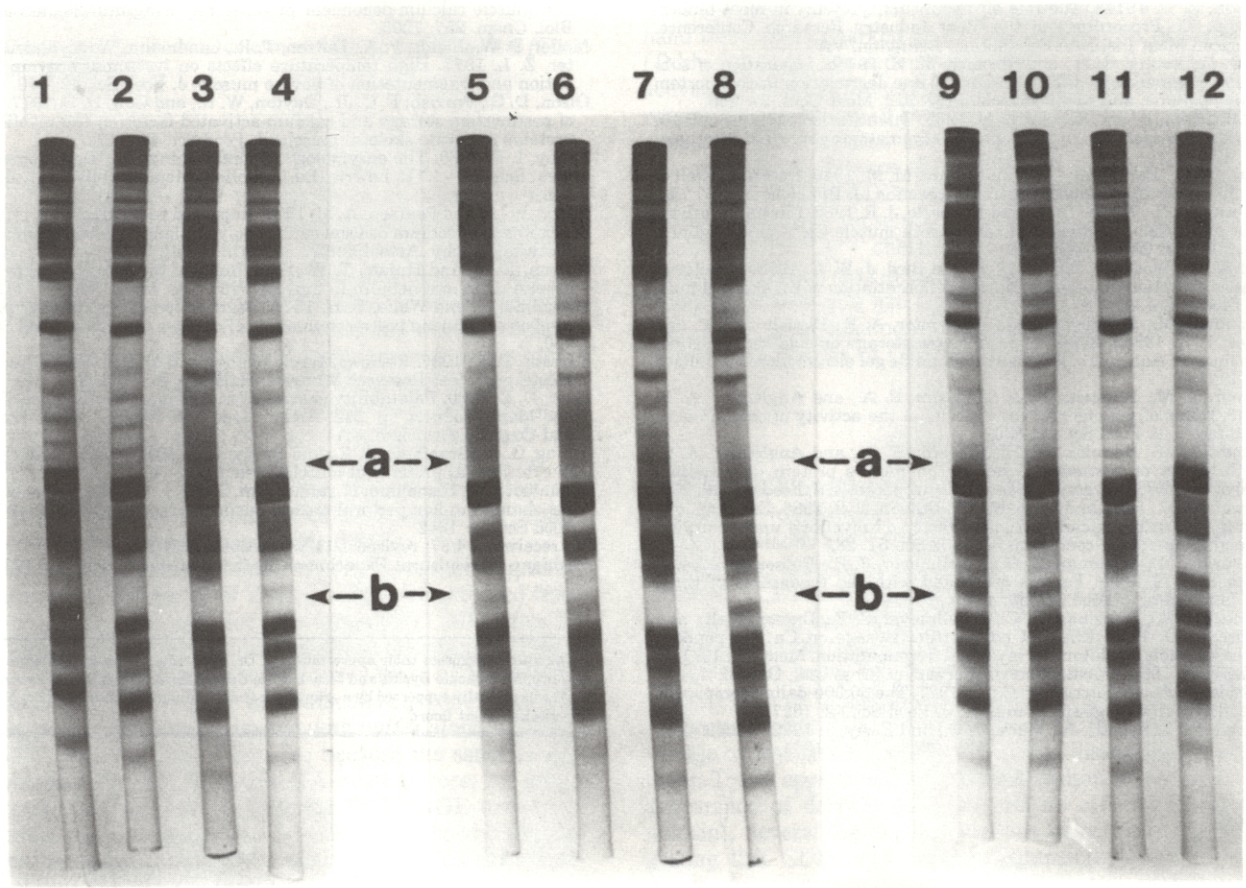

Fig. 3—SDS-PAGE of control samples and slices incubated with buffer alone (100 mM tris-acetate pH 7.2, containing 60 mM Kcl, 1 mM deoxycholate and 1 mM NaN_3), buffer + 10 mM EGTA and buffer + 10 mM $CaCl_2$. Gels 1, 5 and 9 represent control after 1, 3 and 7 days postmortem, respectively. Gels 2, 6 and 10 represent buffer along after 1, 3 and 7 days of incubation at 2–4°C, respectively. Gels 3, 7 and 11 represents buffer + 10 mM EGTA after 1, 3 and 7 days of incubation at 2–4°C, respectively. Gels 4, 8 and 12 represent buffer + 10 mM $CaCl_2$ after 1, 3 and 7 days of incubation at 2–4°C, respectively. a: desmin; b: 30,000 dalton component.

Table 2—Effect of treatments on lysosomal enzyme activities (β units/mg/min)[c]

	Cathepsin B		Cathepsin H		Cathepsin L	
	US	S	US	S	US	S
CONTROL (0 hr)	25.9 ± 5.7	6.9 ± 1.0	121.9 ± 63.6	8.0 ± 3.5	26.5 ± 3.7	8.4 ± 1.0
CONTROL (12 hr)	26.0 ± 5.7	10.8 ± 1.5	136.3 ± 61.4	11.1 ± 4.9	25.9 ± 1.5	11.5 ± 1.3
CONTROL (day 1)	21.1 ± 2.5	9.5 ± 1.7	95.8 ± 32.3[a]	7.7 ± 2.0	25.1 ± 2.9[a]	14.5 ± 1.3
Buffer	32.4 ± 6.5	7.9 ± 1.8	138.3 ± 49.3[ab]	6.8 ± 3.3	38.2 ± 8.5[b]	14.0 ± 1.6
EDTA	29.4 ± 7.5	16.1 ± 6.6	96.2 ± 33.1[a]	5.3 ± 1.0	35.1 ± 11.1[ab]	13.2 ± 1.2
EGTA	31.1 ± 7.2	7.8 ± 2.7	73.5 ± 31.7[a]	4.4 ± 1.1	39.2 ± 9.7[b]	13.6 ± 1.4
$CaCl_2$	28.1 ± 0.9	7.9 ± 3.1	158.6 ± 55.1[b]	6.3 ± 1.5	27.7 ± 5.0[ab]	13.2 ± 1.7

[a,b] Means in a same column with different superscript differ (P<0.05).
[c] Mean ± SEM. US = unsedimentable fraction; S = sedimentable fraction.

Table 3—Effect of treatments on Ca^{2+}-dependent proteases activity (A278/100g muscle)[d]

	0 hr	12 hr postmortem	24 hr post-treatment
CONTROL	55.4 ± 1.4	49.9 ± 2.9	34.8 ± 2.1[b]
Buffer	–	–	39.6 ± 0.4[b]
Buffer + EDTA	–	–	52.2 ± 3.0[a]
Buffer + EGTA	–	–	53.4 ± 4.2[a]
Buffer ± $CaCl_2$	–	–	17.6 ± 1.1[c]

[a,b,c] Means in a same column with different superscript differ (P<0.05).
[d] Mean ± SEM.

postmortem tenderization of meat is to examine the reproducibility of these data in intact carcasses. If the observations with muscle slices occur in meat animal carcasses, the acceptable tenderness of meat from these carcasses can be assured even within 24 hr after slaughter.

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